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(54) Title: METHOD FOR DETECTING EARLY PREGNANCY FACTOR (EPF) IN MAMMALS, PURIFYING EPF AND METHOD FOR PRODUCING A MONOCLONAL ANTIBODY

(57) Abstract

Cells which produce EPF are grown in a culture medium to produce a supernatant medium containing the EPF. To purify the EPF, the EPF is absorbed by a selective absorbent in a column, dialysed against a buffer solution, concentrated and gel-filtered. Selected fractions of the filtrate undergo reversed phase high performance liquid chromotography and the purified EPF is eluted from the chromotography column. Monoclonal antibodies to EPF can be produced to detect the presence of EPF in serum and provide a means for detecting pregnancy in female mammals.

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Title: "METHOD FOR DETECTING EARLY PREGNANCY FACTOR (EPF) IN MAMMALS, PURIFYING EPF AND METHOD FOR PRODUCING A MONOCLONAL ANTIBODY"

BACKGROUND OF THE INVENTION

(1) Field of the Invention

This invention relates to a method for detecting early pregnancy factor (EPF) in mammals, purifying EPF and a method for producing a monoclonal antibody therefor.

(2) Prior Art

Most home pregnancy kits can only detect and indicate pregnancy approximately 3-4 weeks after fertilization.

Pregnancy involves two early important milestones - fertilization of the ovum and implantation of the fertilized ovum in the uterus approximately eight-ten days after fertilization.

It would greatly assist research if the precise times that both milestones occurred could be detected. It would also be an advantage if a woman was aware she was pregnant immediately after fertilization so that she could avoid e.g. smoking, alcohol, surgery and x-rays or radiation treatment.

It has been established that EPF is produced within 24 hours of fertilization but the problem has been to isolate the EPF protein, purify it and produce an antibody which can be used to detect the presence of EPF e.g. in serum or urine as a test for pregnancy.

BRIEF SUMMARY OF THE INVENTION

With the above matter in mind, preferred objects of the present invention are to achieve methods to overcome each of the problems hereinbefore described.

In one aspect the present invention resides

in a method for producing EPF from any mammalian cell source including the steps of:

growing a selected cell which produces EPF in a culture medium to produce a supernatant medium containing EPF and other products; and

harvesting the supernatant medium to obtain the EPF.

In a second aspect the present invention resides in a method for purifying EPF including the steps of:

passing a supernatant medium containing EPF through a column containing a selective absorbent for the EPF (i.e. immuno absorption of the EPF);

eluting the EPF from the selective absorbent to produce a first eluate;

effecting reversed phase high performance liquid chromotography (HPLC) on the first eluate in a column; and

eluting the bound EPF from the column to collect the purified EPF.

Preferably the first eluate is dialysed against a buffer solution to remove any small molecular weight products;

the dialysis product is concentrated;

gel filtration is effected on the concentrate; and

selected fractions of the filtrate are collected and the reversed phase high performance chromotography is effected on the collected fractions.

In a third aspect the present invention resides in a method for producing monoclonal antibodies to EPF including the steps of:

immunizing an animal with purified EPF;

removing the spleen of the animal and fusing the spleen cells with selected cells;

growing the fused cells in a culture medium;

selecting the hybrid (fused) cells from the non-fused cells; and

cloning out the hybrid cells producing the EPF antibody by limiting dilution methods.

The hybrid cells may be grown in a culture medium (i.e. in vitro) to produce high concentrations of monoclonal antibody to EPF, or in BALB/C mice (i.e. in vivo) and harvesting from these mice serum and/or ascites which will contain high concentrations of monoclonal antibody to EPF.

In a fourth aspect the present invention resides in a method for pregnancy diagnosis in a female mammal including the steps of:

mixing EPF antibodies with serum or urine believed to contain EPF; and

monitoring any reaction due to the presence of EPF in the serum or urine.

DETAILED DESCRIPTION OF A PREFERRED EMBODIMENT

To enable the invention to be fully understood, a preferred example will be described with reference to human EPF.

Human EPF was produced by continuously growing Choriocarcinoma cells (sold under the trade mark "Be Wo" by the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. and deposited under ATCC Deposit No. CCL98), human myeloma cells (ATCC Deposit No. CCL155) or human lymphoblastic leukemia cells (ATCC Deposit No. CRL1582) in culture medium comprising "DMEM" ("Dulbecco's Modification of Eagle's Medium") (sold by Flow Laboratories Inc., 7655 Old Springhouse Road, McLean, VA 22102, U.S.A. and subsidiary companies in, inter alia, Australia, Canada, Japan, the United Kingdom and Sweden), and foetal calf serum (sold by Commonwealth Serum Laboratories, Melbourne, Australia) and harvesting the

supernatant medium. This supernatant medium contains human EPF and other products.

An immuno-absorption column is prepared using goat/anti-mouse EPF (or any other suitable EPF e.g. using rabbits or donkeys as the host for mouse, human or rat EPF).

To prepare the column, 800mg. of anti-EPF IgG (immunoglobulin) is absorbed with human serum and with foetal calf serum bound to cyanogen bromide activated Sepharose 4B (sold under the trade name "CNBr-activated Sepharose 4B" by Pharmacia Biotechnology Products, Sweden - see page 91 of their "Catalogue 86") and the absorbed IgG is bound to 30mL of "Affigel-10" (trade name of Bio-Rad, 2200 Wright Avenue, Richmond CA 94804, U.S.A. and subsidiary companies in, inter alia, Australia, Canada, Japan, the United Kingdom and Switzerland. "Affigel-10" is detailed at pages 46-51 of the Bio-Rad Catalogue No. K1985). A pre-column is placed in series with the absorption column having 4g. of IgG from normal male goat serum bound to 100mL. of Affigel-10.

The supernatant medium, containing the EPF, is pumped through the column and the human EPF will bind to the anti-mouse EPF in the absorption column, the latter acting as a selective absorber of the human EPF.

The human EPF is eluted with 1M acetic acid/ 0.9% NaCl/10% dioxane and the eluate is dialysed and the buffer is exchanged for 1M acetic acid adjusted to pH3.0 by ammonium hydroxide to remove small molecular weight products. (All percentages are expressed as "%(w/v) or (v/v)").

The product is concentrated to a 3mL. volume and undergoes gel filtration in a column of Sephacryl S-200 (supplied by Pharmacia Biotechnology Products, Sweden - see page 84 of their"Catalogue 86")which has

been equilibrated with 1M acetic acid adjusted to pH3.0 with ammonium hydroxide.

This sample is filtered on the basis of molecular size and, on a 16mm. x 900mm. column, collecting 2mL. fractions, fractions 60-80 (i.e. 120-160mL. flow) contain the EPF. These are pooled and TFA (trifluroacetic acid) added to a final concentration of 0.1%.

The mixture is applied to a Beckman RPSC ultrapore reversed phase HPLC column (e.g. 4.6mm. x 75mm.) which has been equilibrated with 0.1% TFA.

The bound EPF is eluted with a 2 minute linear gradient to 25% isopropanol followed by a 30 minute linear gradient to 30% isopropanol both containing 0.1% TFA at a flow rate of 1mL./minute.

With the inclusion of a 2mL sample loop in the solvent path of the columns, the fractions eluted with retention times between 9.8 - 11.3 minutes contain the purified EPF. These fractions are pooled and stored. With some immunoabsorbents, it is possible to omit the dialysis and gel filtration steps and apply the eluate directly to the reversed phase HPLC column (which has been equilibrated with 0.1% TFA), after addition of TFA to a final concentration of 0.1%.

To produce the monolconal antibodies to the human EPF, BALB/c mice (e.g. bred from BALB/cJ strain mice from The Jackson Laboratory, Bar Harbor, Maine, 04609, U.S.A.) were immunized with the purified EPF e.g. with 4 to 5 injections at monthly intervals.

The spleens were removed and the spleen cells were fused with mouse myeloma cells (e.g. catalogue Nos. X63-Ag8-653 or Sp2/0-Ag-14 from Flow Laboratories Australasia Pty. Ltd., 140 Wicks Road, North Ryde, Sydney, N.S.W. 2113, Australia).

The cells are grown in "DMEM" medium with

2mM fresh L-glutamine,20% foetal calf serum plus antibiotics and fungicides, and the hybrid cells selected by addition of HAT medium (containing 10^{-4}M hypoxanthine/4 x 10^{-7}M aminopterin/1.6 x 10^{-5}M Thymidine).

The hybrid cells are cloned out by limiting dilution techniques and are tested to establish which clones produce an antibody to EPF. As hereinbefore described, the hybrid cells may be grown in a culture medium (i.e. in vitro) to produce high concentrations of monoclonal antibody to EPF, or in BALB/c mice (i.e. in vivo) and harvesting from these mice serum and/or ascites which will contain high concentrations of monoclonal antibody to EPF.

Those cells are recloned until a cell producing a monoclonal antibody is achieved. Samples of clones in intermediate or final stages are stored in liquid N_2.

The resultant product can be used to detect EPF in human serum or urine for pregnancy diagnosis.

A number of alternative methods for diagnosing pregnancy through the detection of EPF in serum will now be described.

In a liquid phase method, EPF (including some labelled with [^{125}I] is mixed with anti-EPF antibody and the mixture is allowed to react to produce a complex. The complex is precipitated by adding a precipitating antibody or polyethylene glycol.

The presence of any Iodine 125 in the precipitate is monitored using a γ-counter.

A negative sample (i.e. no EPF in the serum) plus the labelled EPF and the antibody will result in a high γ count while a positive sample (containing EPF) plus the labelled EPF and the antibody will result in a low γ count. This is due to competitive binding

between the EPF and labelled EPF with the antibody, as the EPF will prevent the labelled EPF from binding.

In a solid phase method, two different antibodies to EPF may be used, the antibodies binding to different sites on the EPF molecule. One antibody is placed in a plastic tube or on polystyrene beads or sticks and allowed to bind. The serum is added to the tube or placed in contact with the beads or sticks and EPF therein is allowed to bind with the first antibody. A second antibody, labelled with Iodine 125, is then allowed to bind with EPF. The bound Iodine 125 is counted with a γ-counter and a high count indicates the presence of EPF.

This method would be particularly suitable for a home pregnancy testing kit where the first antibody is bound on a testing stick which is dipped into the female's urine specimen and then into a first container supplied with the kit containing the second antibody labelled with an enzyme which undergoes a colour change when the stick dipped into a second container supplied with the kit containing a suitable substrate.

It will be readily apparent to the skilled addressee that the embodiments described above are specific and that a range of chemical proportions and times may be used. For example, the gel filtration may be carried out using "Sephadex G-100" (see page 80 of the Pharmacia Biotechnology Products "Catalogue 86") equilibrated with 1M acetic acid pH2.5.

While human EPF has been described, the methods are suitable for the EPF of all mammalian animals. In particular the method for detecting pregnancy can be extremely important in the horse and cattle industries and in the preservation of endangered species. For example, the giant panda gives no indication of pregnancy but pregnancy could be determined, with-

out handling the female, by collecting urine e.g. from the cage floor, and assaying with the particular suitable monoclonal antibody.

Various changes and modifications may be made to the embodiments described without departing from the scope of the present invention defined in the appended claims.

CLAIMS

1. A method for producing early pregnancy factor (EPF) from any mammalion cell source characterised by the steps of:

growing a selected cell which produces EPF in a culture medium to produce a supernatant medium containing EPF and other products; and

harvesting the supernatant medium to obtain the EPF.

2. A method according to claim 1 characterized in that:

to produce human EPF, the selected cells are choriocarcinoma cells, human myeloma cells, human lymphoblastic cells or a combination of two or more of these.

3. A method according to claim 1 characterized in that:

to produce human EPF, the culture medium comprises "Dulbecco's Modification of Eagle's Medium" and foetal calf serum.

4. A method for purifying EPF wherein the step of harvesting the supernatant medium to obtain the EPF in claim 1 is characterized by the steps of:

passing the supernatant medium through a column containing a selective absorbent for the EPF;

eluting the EPF from the selected absorbent to produce a first eluate;

effecting reversed phase high performance liquid chromotography on the first eluate in a column; and

eluting the bound EPF from the column to collect the purified EPF.

5. A method according to claim 4 characterized in that:

the first eluate is dialysed against a buffer solution to remove any small molecular weight products;

the dialysis product is concentrated;

gel filtration is effected on the concentrate; and

selected fractions of the filtrate are collected and the reversed phase high performance chromotography is effected on the collected fractions.

6. A method according to claim 4 characterized in that:

for producing human EPF, goat/anti-mouse EPF, goat/anti-human EPF, goat/anti-rat EPF, rabbit/anti-human EPF, rabbit/anti-rat EPF, donkey/anti-mouse EPF, donkey/anti-human EPF, donkey/anti-rat EPF, mouse/anti-human EPF, or a combination of two or more of these is used as the selective absorbent for the EPF.

7. A method according to claim 4 characterized in that:

for producing human EPF, the human EPF is eluted with 1M acetic acid/0.9% NaC1/10% dioxane and the buffer solution is exchanged for 1M acetic acid adjusted to pH3.0 by ammonium hydroxide.

8. A method according to claim 5 characterized in that:

the gel filtration is effected in a column containing a gel filtrate equilibrated with 1M acetic acid adjusted to pH3.0.

9. A method according to claim 4 characterized in that:

the selected filtration fractions are pooled and trifluroacetic acid is added to a concentration of 0.1%, and applied to a Beckman C3 reversed phase HPLC column which has been equilibrated with 0.1% trifluroacetic acid.

10. A method according to claim 4 characterized in that:

the bound EPF is eluted with a 2 minute linear gradient to 25% isopropanol followed by a 30 minute linear gradient to 30% isopropanol both containing 0.1% trifluroacetic acid at a flow rate of 1mL per minute.

11. A method according to claim 9 characterized in that:

with the inclusion of a 2mL sample coop in the solvent path of the column, the fractions eluted with retention times between 9.8 - 11.3 minutes contain the purified EPF and these fractions are pooled and stored.

12. A method according to claim 4 characterized in that:

trifluroacetic acid to a final concentration of 0.1% is added to the first eluate before the chromotography step; and

the chromotography step is effected in a Beckman reversed phase HPLC column which has been equilibrated with 0.1% trifluroacetic acid.

13. A method for producing monoclonal antibodies to EPF characterized by the steps of:

immunizing an animal with purified EPF obtained by the method of claim 4;

removing the spleen of the animal and fusing the spleen cells with selected cells;

growing the fused cells in a culture medium;

selecting the fused cells from non-fused cells in a medium; and

cloning out the hybrid cells producing the EPF antibody by limiting dilution methods.

14. A method according to claim 13 characterized in that:

the hybrid cells are grown in vitro in a culture medium to produce high concentration of monoclonal antibody to EPF.

15. A method according to claim 3 characterized in that:

the hybrid cells are grown in vivo in mice and are harvested from the mice serum and/or ascites which contains high concentration of monoclonal antibody to EPF.

16. A method according to claim 13 characterized in that:

the animals are mice which are immunized with 4 to 5 injections at monthly intervals.

17. A method according to claim 13 characterized in that:

the selected cells are mouse myeloma cells;

the culture medium comprises "Dulbecco's Modification to Eagle's Medium",20% foetal calf serum, antibiotics and fungicides; and

the hybrid cells are selected in a HAT medium to kill any background cells.

18. A method according to claim 17 characterized in that:

the cloned hybrid cells are tested to establish which clones produce an antibody to EPF; and

these cells are recloned until a cell producing a monoclonal antibody is achieved.

19. A method for diagnosing pregnancy in a female mammal characterized by the steps of:

mixing antibodies produced by the method of claim 13 with serum or urine of the femal mammal believed to contain EPF; and

monitoring any reaction due to the presence of EPF in the serum or urine, the presence of EPF indicating pregnancy in the female mammal.

20. A method according to claim 19 characterized in that:

the EPF in the serum or urine is used with an

anti-EPF antibody with the addition of a trace amount of purified EPF labelled with Iodine 125;

the mixture is allowed to react to form a complex;

the complex is preciptiated out; and

the presence of any Iodine 125 in the precipitate is monitored using a γ-counter, a low γ count indicating the presence of EPF in the serum or urine.

21. A method according to claim 19 characterized in that:

a first anti-EPF antibody is bound to a plastic tube, stick or beads;

the serum or urine is placed in contact with the first antibody to enable any EPF in the serum or urine to bind with the antibody;

a second anti-EPF antibody, labelled with Iodine 125, is placed in contact with the EPF to bind therewith; and

the presence of bound Iodine 125 is monitored with a γ-counter, a high count indicating the presence of EPF.

22. A method according to claim 19 characterized in that:

a first anti-EPF antibody is bound to a plastic stick;

the stick is dipped in the serum or urine to enable any EPF therein to bind with the first antibody; and

the stick is dipped into a second anti-EPF antibody labelled with an enzyme and then a substrate which undergoes a colour change if EPF is present in the serum or urine.

23. Purified EPF obtained by the method of any one of claims 4 to 12.

24. Monoclonal antibodies to EPF obtained by the method of any one of claims 13 to 18.

25. A pregnancy testing kit for female mammals characterized by:

a plastic tube container, stick or beads to which are bonded, a first anti-EPF antibody;

a first container containing a second anti-EPF antibody labelled with an enzyme; and

a second container containing a substrate, wherein:

after serum or urine from the female mammal is placed in the tube, or the stick or beads are dipped in the serum or urine from the female mammal, to enable any EPF therein to bind with the first anti-EPF antibody; and

the second anti-EPF antibody and then the substrate are placed in the tube, or the stick or glass beads are dipped in the second anti-EPF antibody and then the substrate, a change of colour of the substrate indicates the presence of EPF in the serum or urine and that the female mammal is pregnant.

AMENDED CLAIMS

[received by the International Bureau on 14 August 1986 (14.08.86); original claims 1,3 and 25 amended; other claims unchanged (2 pages)]

1.(Amended) A method for producing early pregnancy factor from any mammalian cell source characterized by the steps of:

growing in vitro a selected cell line which produces EPF in a culture medium to produce a supernatant medium containing EPF and other products; and

harvesting the supernatant medium to obtain the EPF.

2. A method according to Claim 1 characterized in that:

to produce human EPF, the selected cells are choriocarcinoma cells, human myeloma cells, human lymphoblastic cells or a combination of two or more of these.

3.(Amended) A method according to Claim 1 characterized in that:

to produce human EPF, the culture medium comprises "Dulbecco's Modification of Eagle's Medium" and/or foetal calf serum.

4. A method for purifying EPF wherein the step of harvesting the supernatant medium to obtain the EPF in Claim 1 is characterized by the steps of:

passing the supernatant medium through a column containing a selective absorbent for the EPF;

eluting the EPF from the selected absorbent to produce a first eluate;

effecting reversed phase high performance liquid chromotography on the first eluate in a column; and

eluting the the bound EPF from the column to collect the purified EPF.

5. A method according to Claim 4 characterized in that:

the stick is dipped in the serum or urine to enable any EPF therein to bind with the first antib dy; and

the stick is dipped into a second anti-EPF antibody labelled with an enzyme and then a substrate which undergoes a colour change if EPF is present in the serum or urine.

23. Purified EPF obtained by the method of any one of the Claims 4 to 12.

24. Monoclonal antibodies to EPF obtained by the method of any one of Claims 13 to 18.

25.(Amended) An early pregnancy testing kit for female mammals to detect the presence of EPF in serum or urine characterized by:

a plastic tube container, stick or beads to which are bonded, a first anti-EPF antibody;

a first container containing a second anti-EPF antibody labelled with an enzyme; and

a second container containing a substrate, wherein:

after serum or urine from the female mammal is placed in the tube, or the stick or beads are dipped in the serum or urine from the female mammal, to enable any EPF therein to bind with the first anti-EPF antibody; and

the second anti-EPF antibody and then the substrate are placed in the tube, or the stick or glass beads are dipped in the second anti-EPF antibody and then the substrate, a change of colour of the substrate indicates the presence of EPF in the serum or urine and that the female mammal is pregnant.

INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 86/00060

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[4] C07K 15/06, 15/12, 3/20, C12N 15/00, G01H 33/577

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC	C07G 7/00, C07K 15/06
US Cl.	260-112 R

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

AU : C07G 7/00 (010, 011, 014, 101), C07K 15/06

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Journal of Reproduction and Fertility, Volume 71, Number 2, issued 1984 (University of Queensland, Australia), Alice C. Cavanagh, Production In Vitro of Mouse Early Pregnancy Factor and Purification to Homogeneity, see pages 581-92	1-12
X,Y	Reproductive Immunology, Proceedings of 2nd International Congress, Published 1983 by Elsevier Science Publishers (Amsterdam), Studies on Human Early Pregnancy Factor, pages 157-69, by Timothy K. Roberts and Cheng Y. Smart	1-12,25
X	Pregnancy Proteins, Biological, Chemical Clinical Applications, Published 1982 by Academic Press (Australia) (Conference Proceeding), Frank Clark et al, Biochemistry of Early Pregnancy Factor, pages 407-412	1-12
X	Journal of Reproductive Immunology, Volume 5 Number 5 1983 (Griffith Univ., Australia) Shann Wilson et al, In Search of Early Pregnancy Factor : isolation of active polypeptides from pregnant ewe's sera, pages 275-86	1-12

CONTINUED

* Special categories of cited documents: [10]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
10 June 1986 (10.06.86)	(17.06.86) 17 JUNE 1986
International Searching Authority	**Signature of Authorized Officer**
Australian Patent Office	J.P. PULVIRENTI

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	Journal of Reproductive Immunology, Volume 6 Number 4 1984 (Griffith Univ., Australia) Shann Wilson et al, In Search of Early Pregnancy Factor : characterization of active polypeptides isolated from pregnant ewe's serum, pages 253-60	1-12
X	Pregnancy Proteins: Biological, Chemical Clinical Applications, Published 1982 by Academic Press (Australia) (Conference Proceeding) Halle Morton et al, Early Pregnancy Factor : biology and clinical significance, pages 391-405	1
Y	GB,A, 1563299 (RAFA LABORATORIES LTD) 26 March 1980 (26.03.80)	25
A	Journal of Reproduction and Fertility, Volume 69, Number 2, 1983 (Institut Fur Tierzucht und Tieverhalten, Neustadt, West Germany) Halle Morton et al, The Appearance and Characteristics of Early Pregnancy Factor in the Pig, pages 437-46	

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 86/ 00060

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Members		
GB 1563299	AT 9621/76	AU 20495/76	BE 849667
	DE 2657292	ES 45442	FI 763647
	FI 763647	FR 2336113	IL 48741
	IL 48741	IT 1068738	JP 52082892
	NL 7614359	SE 7614387	

END OF ANNEX